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The Systematics and Bionomics of Malaria Vectors in the Southwest Pacific

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1. Introduction

1.1. Malaria in the Southwest Pacific

The malaria transmission zone in the southwest Pacific ranges from Indonesia (Papua Province) through Papua New Guinea (PNG) and the Solomon Islands to Vanuatu. The island of Tanna in Vanuatu marks the southern and eastern limit of the region's malaria endemic area. The malaria-free island of Aneityum is the most easterly location where anophelines are found (Fig 1). While northern Australia previously experienced regular outbreaks of malaria, the disease was eliminated in 1962 [1] – although it still experiences sporadic outbreaks following reintroductions of the parasites [2]. Malaria remains the most important vector-borne disease in the region with Indonesian Papua, PNG and the Solomon Islands enduring some of the highest attack rates in the world outside Africa [3].

Malaria is endemic below 1000m, with the degree of endemicity ranging from hypoendemic to holoendemic [4, 5]. Above 1000m malaria tends to be unstable with epidemics of varying degrees of severity [6-8]. Serious control efforts were initiated in the 1950s-1960s as part of the WHO Global Eradication Program, with pilot projects implemented in Papua Province (Indonesia) and PNG (late 1950s) and in the Solomon Islands and Vanuatu (late 1960s). The principal strategy was indoor residual spraying (IRS) with DDT supplemented with mass drug administration of chloroquine [9].

In 1969, the malaria eradication was abandoned in Papua Province and PNG as it was realized that this goal was not attainable – instead, various control programs were introduced. In PNG, IRS continued until 1984, after which little more was done in the way of malaria vector control until the early 1990s, when insecticide treated bed nets (ITNs) were trialed [10] prior to

widespread distribution. In the Solomon Islands and Vanuatu, full-scale malaria eradication programs (MEP) commenced in the early 1970s but were also abandoned after three years and replaced with control programs [11]. In both countries pyrethroids replaced DDT in IRS in the early 1990s and ITNs became the main method of control [12]. During the 1990s, malaria was successfully eliminated on Aneityum Island, the most southern island of Vanuatu [13] with mass drug administration as the primary intervention. Recently, renewed efforts at malaria elimination and intensified control were initiated in Tafea Province in Vanuatu and Temotu and Santa Isabel Provinces in the Solomon Islands [14].

1.2. Geography and climate

This work covers the malarious area of the southwest Pacific as it lies within the Australian faunal region (Fig. 1). This region is made up of numerous islands many of which are mountainous (>4000m) with ranges extending to the coasts and drained by river systems over a narrow coastal plain. In New Guinea, the ranges are fragmented by river valleys, creating extensive lowlands comprising flood plains and swamps. Throughout the region, the climate is dominated by two wind systems and by the influence of mountain barriers and the surrounding oceans. From December to April (the wet season), moist northwesterly winds produce the heaviest and most frequent rains. From May to October (the dry season), southeasterly winds prevail and conditions are drier. However during this period substantial rainfall occurs wherever prominent mountain barriers exist. Thus the climate for most of the region is continuous hot/wet with rainfall >2000mm p.a. with rainless periods rarely exceeding four days. Exceptions occur in southern Western Province and around Port Moresby in PNG where the climate is more monsoonal, the dry season is more pronounced, and the rainfall is less (1600-2000mm p.a.) (Fig. 1) [15].

Temperature is not a major climatic factor as there is little seasonality and minimal variation throughout each year in a given elevation. However, elevation exerts the main influence on temperature: in coastal and lowland areas (<500m), the mean temperature is 26°C (max 31°C; min 22°C), while in the highland regions (>500m), the mean temperature is 20°C (max 23°C; min 14°C) [15].

2. Systematics of the malaria vector Groups

The anopheline fauna of the Australian Region is delimited in the west by the Weber Line, which runs through the Moluccas, though there is some incursion east and west of this line by anophelines from the Oriental and Australian Regions (Fig 1 and Table 1). The Australian fauna is highly endemic and most likely of Oriental origin. The malaria vectors in the Australian Region are composed of groups and complexes of closely related, morphologically similar, cryptic or sibling anopheline species. Accurate identification of vector species is essential for interpreting the efficacy of interventions in an area. Since the discovery of cryptic sibling species, the use of morphological characters previously used to identify species has been rendered uncertain. Techniques such as cross-mating, chromosome studies and allozyme

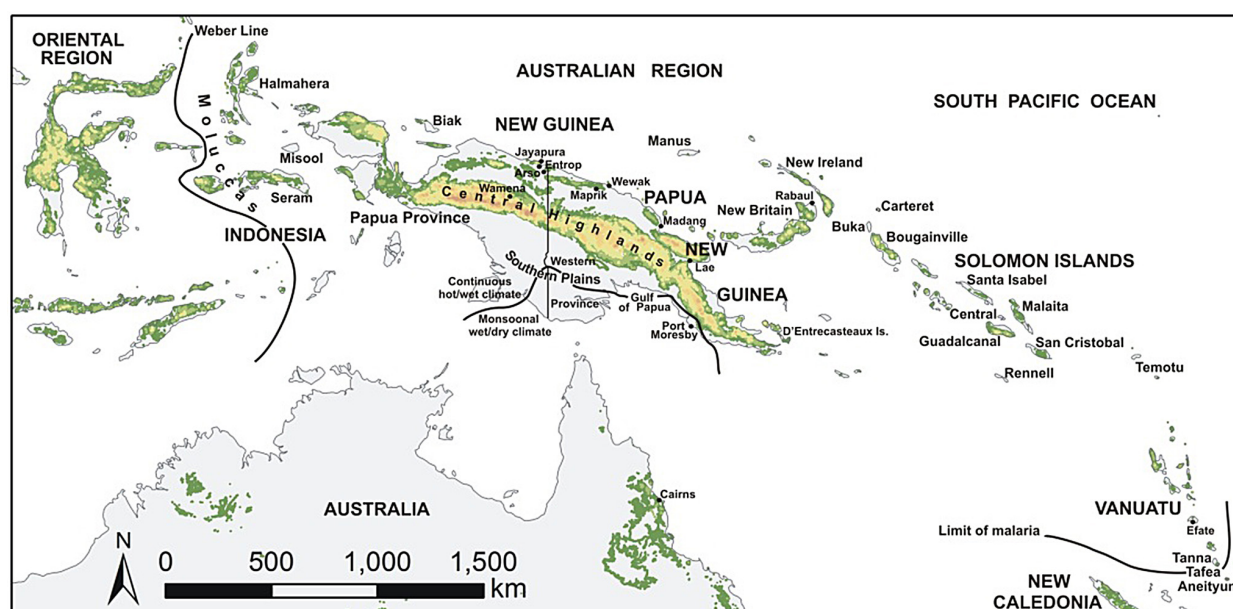


Figure 1. Map of the southwest Pacific region showing regions and sites described in the text. The malaria vectors described in this chapter exist from the Moluccas in the west (approximately at the Weber line) to Vanuatu in the east and south into northern Australia. Note: The green to orange shading represents elevation from 600m to 4,800m.

analysis were initially deployed to resolve the problems of identifying these sibling species, though none of these can match the speed and simplicity of morphological markers which could be applied in the field. Advances in DNA-based technology with high throughput capability during the past two decades allow large and detailed analyses of vector populations. Although more costly and requiring sophisticated laboratory support, methods such as DNA probe hybridization and PCR are both quick, user-friendly and offer advantages in the study of intraspecific differences between species and for phylogenetic studies. Studies of the *Anopheles punctulatus* group of the southwest Pacific provides a prime example of both the application of this technology and how it has progressed.

Because of advances in DNA-based technologies, mosquito taxonomists and systematists can now identify, describe, and classify *Anopheles* biodiversity, in addition to studying and understanding their evolution, distribution, and species' relationships. The practical relevance of such information extends beyond the labeling and ordering of taxa. Studies of malaria transmission reinforce time and again the importance of incorporating an intimate knowledge of *Anopheles* species biology, behavior, and ecology into the design, implementation and evaluation of any successful vector control strategy. Control strategies require information on vector species distribution, their density, and seasonal prevalence as well as data on mating, oviposition, feeding and resting habits, longevity and fecundity, and susceptibility to both parasites and insecticides. Yet measurements of these entomological parameters are only relevant if accurate vector species' identifications are possible. Each species has evolved characteristics that will influence its ability to transmit malaria and its vulnerability to any control strategies depends on these behavioural characteristics. Additionally, systematics and phylogeny can provide useful information on host/parasite evolution, ecological adaptation,

Species, Groups, and Complexes	Moluccas	New Guinea ¹					Solomon Islands	Vanuatu	Vector status
		Monsoo- nal	Hot / wet	High- lands	SCH	NCH			
Subgenus <i>Anopheles</i>									
<i>An. bancroftii</i> complex four species A-D		xxx ²	xxx	xx	xxx	xxx			secondary
<i>An. papuensis</i>				x					non-vector
Subgenus <i>Cellia</i>									
<i>An. annulipes</i> complex:									
<i>An. annulipes</i> L		xxx			x				non-vector
<i>An. annulipes</i> M				xxx					non-vector
<i>An. hilli</i>		xxx							possible
<i>An. karwari</i> (Oriental)	xx					xxx			secondary
<i>An. longirostris</i> complex: nine species 1-9			xxx		xxx	xxx			secondary
<i>An. lungae</i> complex:									
<i>An. lungae</i>							xxxx		possible
<i>An. solomonis</i>							xxxx		possible
<i>An. nataliae</i>							xxxx		possible
<i>An. meraukensis</i>		xx							possible
<i>Annovaguinensis</i>		xx							possible
<i>An. punctulatus</i> group:									
<i>An. farauti</i> complex:									
<i>An. farauti</i>	xxx	xxxx	xxxx		xxxx	xxxx	xxxx	xxxx	primary
<i>An. hinesorum</i>	x	xxxx	xxxx	x	xxxx	xxx	xxxx		secondary
<i>An. torresiensis</i>		xx							possible
<i>An. farauti</i> 4			xxx			xxx			secondary
<i>An. farauti</i> 5				x					non-vector
<i>An. farauti</i> 6				xxx					secondary
<i>An. irenicus</i>							xxx		non-vector
<i>An. farauti</i> 8			x						secondary
<i>An. clowi</i>			x			x			non-vector
<i>An. koliensis</i>			xxxx		xxxx	xxxx	x		primary
<i>An. punctulatus</i>			xxxx	xx	xxxx	xxxx	xx		primary
<i>An. sp</i> near <i>punctulatus</i>			xx		xx	xx			non-vector
<i>An. rennellensis</i>							x		non-vector
<i>An. subpictus</i> (Oriental)	x	xx	xx		xx	x			possible
<i>An. tessellatus</i> (Oriental)	x		x			x			non-vector

Monsoonal type climate; continuous hot/wet type climate, highlands >300m; SCH: south of the central highlands; NCH: north of the central highlands

xxxx: abundant, xxx: common, xx: uncommon, x: rare

Table 1. The *Anopheles* species currently found in the Australian Region, their distribution and vector status.

and biogeography. The following section outlines our current knowledge of the primary and secondary malaria vectors of the southwest Pacific region.

2.1. The *Anopheles (Cellia) punctulatus* group

The primary vectors of malaria throughout the southwest Pacific region are members of the *Anopheles punctulatus* group. In 1901 Dönitz described the type form [16], *Anopheles punctulatus*, from the Madang area of PNG, while Laveran described *Anopheles farauti* in Efate, Vanuatu, the following year [17]. Given that the range of the *An. punctulatus* group spans several countries, the early identity and relationship of the members was somewhat confused – a detailed account of this early history is given in Lee et al. [18] and Rozeboom and Knight [19].

Thanks in part to the necessary deployment of Allied defense personnel throughout this region; the taxonomy of this vector group was studied in depth during World War II. Four closely related species were identified – *An. punctulatus* Dönitz, *An. farauti* Laveran, *An. koliensis* Owen and *An. clowi* Rozeboom and Knight – and assembled within the Punctulatus Complex [19].

In 1962, Belkin referred to the group in his taxonomic study of South Pacific mosquitoes [20]. However, this study did not include Irian Jaya, Indonesia (now West Papua/Papua Province) or PNG. Rozeboom and Knight [19] provide descriptions of the original four members of the *An. punctulatus* complex and taxonomic keys for the members of the complex. For adult females, the diagnostic characters used were the black and white scaling patterns on the proboscis and, to a lesser extent, on the wings, palpi, and tarsi. Proboscis morphology readily, but unreliably as was later learned, separated the three most common and widespread members, *An. farauti*, *An. punctulatus*, and *An. koliensis*. *Anopheles farauti* displays an all black scaled labium; *An. punctulatus* has the apical half of the labium extensively pale scaled; and *An. koliensis* has a patch of pale scales, varying in size, on the ventral surface of the apical half of the labium [19]. For *An. clowi*, the tarsi on the fore- and mid-legs were used [19].

Taxonomic and systematic studies of the group were renewed in the 1970's when Bryan showed that cross-mating between two *An. farauti* colonies (from Rabaul in PNG and north Queensland) was incompatible as the species differed by two paracentric inversions [21]. The two species were then called *An. farauti* 1 and *An. farauti* 2. Bryan then collected material from the type locality (Efate, Vanuatu) and identified it as *An. farauti* 1 [22], hereafter referred to as *An. farauti*. Hybridization experiments by Mahon and Miethke in 1982 [23] revealed another species (designated *An. farauti* 3) and also found three sympatric sibling species with no evidence of interbreeding in the Innisfail region south of Cairns in north Queensland. Bryan also confirmed the species status of *An. koliensis* in 1973 by cross-mating experiments [24]. Also in 1973, Maffi described specimens from Rennell Island in the Solomon Islands as belonging to the *An. punctulatus* group [25] and subsequently declared these mosquitoes as a new species, *An. rennellensis* [26]. In the late 1980s, *An. farauti* was identified from the coastal areas around Madang, PNG [27], and Sweeney showed that salt tolerance could be used as a species diagnostic feature [28].

Although proboscis markings are often obvious and easy to detect, proboscis morphology is not a reliable means of distinguishing species in this group. As early as 1945, working in PNG, Woodhill [29] examined the progeny of wild caught females of the “intermediate form” (now called *An. koliensis*) and found both *An. farauti*- and *An. punctulatus*-type proboscis. Similar polymorphisms in this character were also noted by Foley et al. [30] and Cooper et al. [31]. Later morphological studies [32, 33] using specimens from Australia and the Solomon Islands described morphological features for *An. farauti* species and provided preliminary keys. However these keys are problematic as the characters used are both difficult for routine identification and are not 100% accurate. In addition, they were developed using material from a limited range of the species’ distributions. Figure 2 and Table 2 summarizes some problems with using proboscis morphology for identifying members of the *An. punctulatus* group.

The *An. punctulatus* group currently consists of 13 species that include: *An. punctulatus*, *An. koliensis*, *An. species near punctulatus*, *An. clowi*, *An. rennelliensis*, and the members of the *An. farauti* complex: *An. farauti* (formally *An. farauti* 1), *An. hinesorum* (formally *An. farauti* 2), *An. torresiensis* (formally *An. farauti* 3), *An. irenicus* (formally *An. farauti* 7) and *An. farauti* 4-6 and 8 [30, 33-37]. Given that the majority of the 13 species currently known in the *An. punctulatus* group were discovered in the 1990's, a great deal of polymorphism can be presumed to exist in the morphological characters previously used to describe the members of this group. As a consequence, field workers who rely on proboscis morphology should also be using the available molecular tools [30, 31, 38-40] (see Fig. 2 and Table 2).

Species (number identified by PCR)	Proboscis Type ¹ number (%)		
	farauti	koliensis	punctulatus
<i>An. farauti</i> (n=1,131)	1,128 (99.7)	0 (0)	3 (0.3)
<i>An. hinesorum</i> (n=1,050)	1,048 (99.8)	1 (0.1)	1 (0.1)
<i>An. farauti</i> 4 (n=842)	235 (28.0)	472 (56.0)	135 (16.0)
<i>An. koliensis</i> (n=1,223)	151 (12.3)	1,035 (84.7)	37 (3.0)
<i>An. punctulatus</i> (n=676)	4 (0.6)	16 (2.4)	656 (97.0)

farauti - all black scaled labium; *koliensis* - dorsal white patch of scales on the anterior end; *punctulatus*: anterior half all white scaled.

Table 2. Proboscis morphology of five common members of the *Anopheles punctulatus* group from the Australian Region and identified using DNA hybridisation and PCR-RFLP analysis.

The distribution of these species is only beginning to be understood as the group ranges over hundreds of small islands with varying landforms and ecotypes, each island providing opportunities for reproductive isolation and consequent speciation. It is possible that further species may be found when the remote and inaccessible areas of the Moluccas, Indonesian Papua, Papua New Guinea, and the Solomon Islands are more thoroughly surveyed.

2.1.1. Molecular genetic markers

After cross-mating experiments revealed post-mating barriers and the presence of the three species designated *An. farauti*, *An. hinesorum*, and *An. torresiensis* [24], mosquito cytogenetics became a more informative and practical method to study and identify these species. In 1971, Bryan and Coluzzi [21] produced preliminary maps of polytene chromosomes from the salivary glands of 4th instar larvae of *An. farauti* and *An. hinesorum*. Taking *An. farauti* as the standard, *An. hinesorum* differed by a paracentric inversion on each of the left and right arms of chromosome 2 [21]. Mahon [41] found that *An. torresiensis* had the standard arrangement for the autosomes but the X chromosome differed by two inversions. The same author also looked at chromosome maps of *An. punctulatus* and *An. koliensis* and predicted chromosomal relationships among the five species and possible ancestral characters [41].

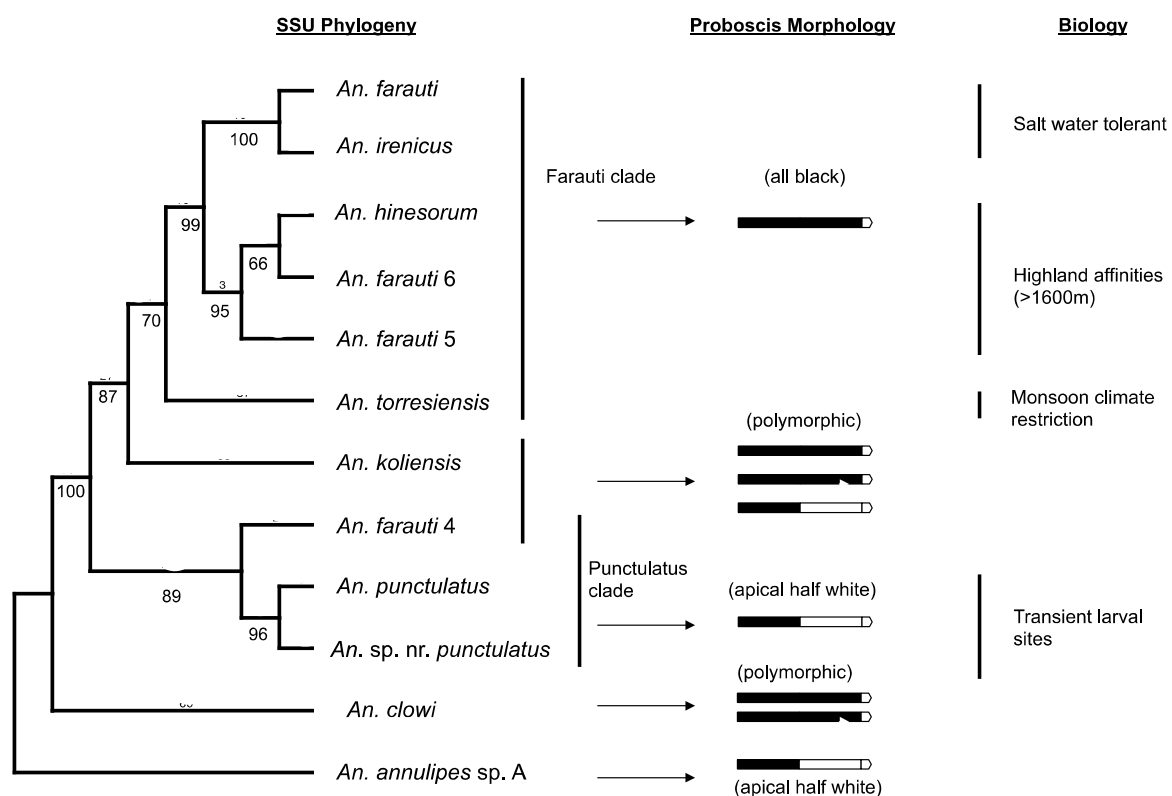


Figure 2. This single most parsimonious phylogenetic tree generated from the structural alignment of the nuclear *ssrDNA* reveals 11 members of the *An. punctulatus* group with *An. annulipes* sp. A from the *An. annulipes* outgroup. Proboscis morphologies identified from field-collected specimens are displayed to the right and overt biological characteristics are also listed.

2.1.2. Molecular markers

Allozymes: In the 1990's Foley and colleagues [30] executed the first population genetic studies into the group using allozyme electrophoresis methods to show that *An. farauti* specimens from inland areas around Madang were reproductively isolated from the PNG highlands. In doing this, they discovered *An. farauti* 4 from the Madang area and *An. farauti* 5 and 6 from the PNG highlands. Then, also using allozymes, Foley revealed a reproductively isolated *An. farauti*-like species from Guadalcanal in the Solomon Islands and designated it *An. farauti* 7 (now *An. irenicus*) [35]. Furthermore, a population with morphology very like *An. punctulatus* was found in the Western Province of PNG and appeared reproductively isolated; this was named *Anopheles* species near *punctulatus* [34].

To facilitate the identification of the large numbers of field-collected material required for malaria studies, Mahon [42] developed a starch gel allozyme electrophoresis method using two enzymes, lactate dehydrogenase and octanol dehydrogenase. This method was employed to study the distribution of cryptic species of *An. farauti* throughout northern Australia [43, 44]. The allozyme technique was further refined with cellulose acetate electrophoresis by Foley in 1993 [30, 45] to also identify *An. farauti* 4, 5, 6, *An. irenicus*, and *An.* species near *punctulatus* [30, 34, 35]. Thus electrophoretic keys were now available for ten species in the *An. punctulatus* group – excluding the rarely recorded *An. clowi* and *An. rennellensis* [34]. These allozyme markers represent the first molecular tools to identify the members of the *An. punctulatus* group. The requirement of a cold (frozen) chain from the field to the lab to prevent protein degradation of samples was the most limiting feature of this technology.

2.1.3. Species-specific genomic DNA probes

Chromosome banding differences discovered while identifying cryptic species revealed a large variations in the genomic DNA of these species, and suggested possible avenues for producing new technologies for identifying cryptic species. Advances in recombinant DNA technology in the early 1980's enabled the isolation of species-specific repetitive DNA sequences. The use of nucleic acids as characters to identify the members of this group began in 1991 with the development of isotopic DNA probes for the Australian species *An. farauti*, *An. hinesorum*, and *An. torresiensis* [46]. Genomic DNA probes were developed for use with squash blot techniques for ten species in the *An. punctulatus* group [38, 46, 47]. The “squash blot” (see Fig. 3 for an example) technique requires no DNA extraction; the specimen (or part of specimens) is squashed directly onto the membrane in the presence of a detergent that ruptures the tissue. The liberated DNA then binds to the nylon membrane. Species-specific probes labeled with a reporter molecule such as biotin or ³²P hybridize to homologous DNA from the squashed material and are visualized by the reporter molecule [46]. Up to 100 membranes can be probed simultaneously, permitting thousands of field specimens to be identified for a particular species. Over 100,000 species identifications were thereby processed to produce the extensive distribution data generated by Cooper and colleagues [31, 44, 48, 49].

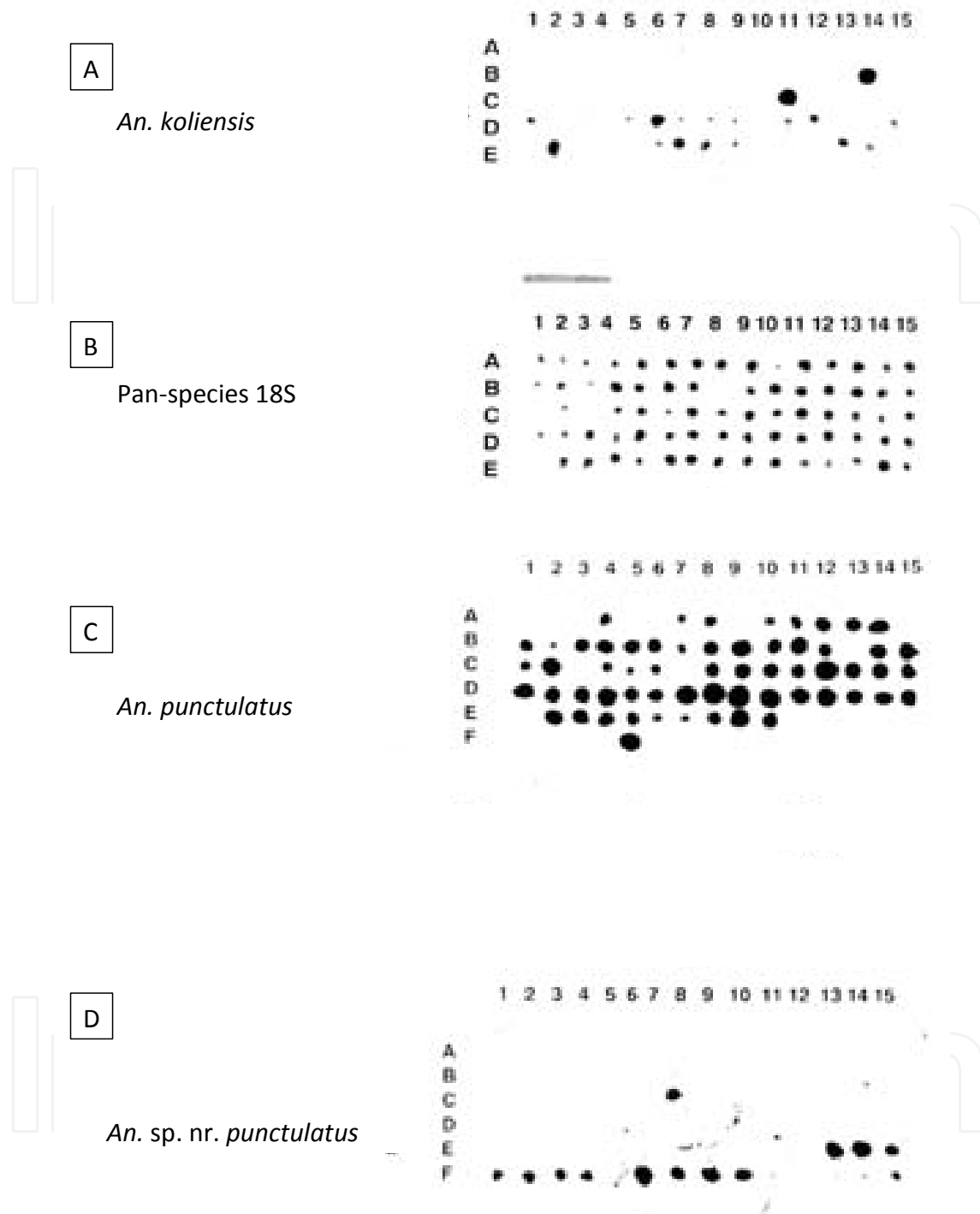


Figure 3. Mosquito squash blots hybridized with species-specific genomic DNA probes labeled with ^{32}P can distinguish cryptic species in the *An. punctulatus* group. **Panel A:** squash blot of mosquitoes morphologically identified as *An. koliensis* and probed with a species-specific probe reveals that only a subset of samples are *An. koliensis* (*An. farauti* 4 made up the other individuals identified as *An. koliensis*). **Panel B:** same blot was stripped and probed with a pan-species rDNA 18S probe that binds to all species revealing the total amount of gDNA on the blot. **Panel C:** mosquitoes identified as *An. punctulatus* are probed with the *An. punctulatus* species-specific probe and **Panel D** is the same blot stripped and reprobed with the *An. sp. nr. punctulatus* probe.

2.1.4. PCR-based species diagnostics

2.1.4.1. Ribosomal DNA ITS2

The advent of polymerization chain reaction (PCR) for DNA amplification in the late 1980's facilitated technologies for both cryptic species' identification and within-species population studies. The most popular marker for species-specific PCR-based diagnosis has been the rDNA gene family. Despite a lack of understanding of the evolution of this non-Mendelian evolving repetitive gene family, its rapidly evolving transcribed spacers allow a simplistic evaluation of genetic discontinuity within and between species. The internal transcribed spacer 2 (ITS2) region proved the most useful for developing two different species diagnostic tools for identifying *An. punctulatus* group members [40, 50]. In the first PCR-RFLP (restricted fragment length polymorphism) technology, the size of the ITS2 region (~710bp) was identical for all *An. punctulatus* group members and was thus diagnostic for the group; this means that mosquito collections of other (non-*An. punctulatus* group) species can be detected simply as RFLPs of different banding profiles. Digestion of this product with the restriction enzyme *Msp* I generates species-specific DNA fragments for the 11 most abundant and most widely distributed members of this group, *An. farauti*, *An. hinesorum*, *An. torresiensis*, *An. farauti* 4-6, *An. irenicus*, *An. punctulatus*, *An. species near punctulatus*, and *An. clowi* (Fig. 4). This species-specific PCR-RFLP has been extensively used both independently and alongside genomic DNA probes in species distribution studies of the *An. punctulatus* group [31, 44, 48, 51]. However, more recently, a "Luminex®"-based multiplex ligase detection reaction and fluorescent microsphere-based assay method became available, also based on species-specific ITS2 sequences, and can separate the five common malaria vector species in PNG: *An. punctulatus*, *An. koliensis*, *An. farauti*, *An. hinesorum*, and *An. farauti* 4 [40].

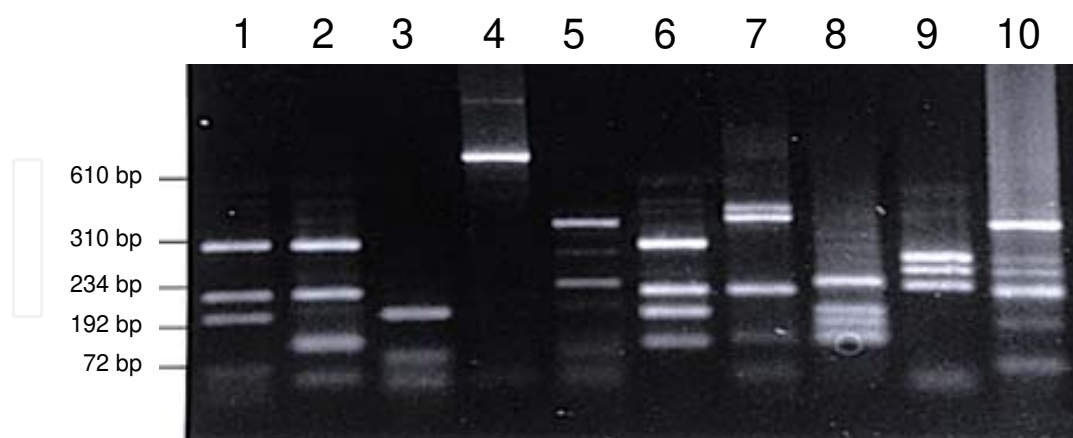


Figure 4. Molecular diagnostic that discriminates over 10 members of the *An. punctulatus* group based on a PCR-RFLP of the ITS2, cut with the restriction enzyme *Msp* I and run out on a 3% agarose gel. Banding profiles are as follows: Lane 1, *An. farauti*; (formally *An. farauti* 1) Lane 2, *An. hinesorum* (formally *An. farauti* 2); Lane 3, *An. torresiensis* (formally *An. farauti* 3); Lane 4, *An. farauti* 4 (contains no restriction site); Lane 5, *An. farauti* 5; Lane 6, *An. farauti* 6; Lane 7, *An. irenicus* (formally *farauti* 7), Lane 8, *An. koliensis*, Lane 9, *An. punctulatus*; Lane 10, *An. sp. nr. punctulatus*. Additionally *An. clowi* can be distinguished using this method however *An. farauti* 8 produces the same RFLP profile as *An. farauti*, but is distinguishable by ITS1 RFLP[52].

Analysis of the ITS2 region reveals substantial insertion and deletion events (indels) between species that are probably due to sequence slippage of common, simple, sequence repeat motifs. Interestingly, no ITS2 PCR-RFLP mixed species hybrids have yet been reported, which would be observed as single mosquitoes sharing RFLP profiles of more than one species. The lack of hybrids at the rDNA locus reinforces the species status for members of this group. Additionally evolutionary information about the *An. punctulatus* group has been obtained with studies of the ITS2 region. The undigested ITS2 PCR products from single mosquitoes contain ITS2 sequence copy variants in the multicopy rDNA array and can provide another view on population genetic structure. For example, intraspecific rDNA genotypes of *An. farauti* were found to be geographically structured by the presence of fixed ITS2 copy variants amplified in the PCR [53] (also see Figs. 5, 6, and 7 for examples). Population genetic analyses of *An. farauti* revealed macrogeographic population structure in *An. farauti* throughout the southwest Pacific comprising several distinct genotypes, suggestive of potential barriers to gene flow. Interestingly, only a subset of these geographically structured genotypes were identified at the level of the mitochondrial DNA cytochrome oxidase I (COI) sequence level in a recent population genetic study of this species [54], suggesting that the rDNA array may be a sensitive tool for species-level diagnostics.

While the ITS1 region has not been examined in as much detail as the ITS2, the ITS1 is an informative marker for intraspecific population studies for some *An. punctulatus* group members, separating *An. farauti* into several geographically and climatically distributed genotypes [52, 53]. For example, Fig. 5 shows how the ITS2 and ITS1 can reveal qualitative information on population genetic discontinuities within *An. farauti* where rDNA genotypes could also be identified within and between landmasses reflecting genetic and geographic structure [53]. This phenomena was most likely possible because of the extended time this species existed in a region with natural barriers to gene flow [54].

2.1.5. Evolutionary and phylogenetic studies

Identifying levels of genetic differences among mosquito taxa and the phylogenetic relationships of closely related species allows an understanding of the evolutionary forces acting on mosquito populations. Knowing the evolutionary relationships among vector species can provide insights into understanding the dynamics of disease transmission. Initial attempts to generate a species-level phylogeny of the *An. punctulatus* group were based on the DNA sequence of the rDNA ITS2. However, the large amount of sequence variation between each species appearing as insertion or deletion indels made computer-based sequence alignment difficult, and the resulting systematic trees could not resolve all species in the group [55]. The closely linked ssrDNA (rDNA 18S) structural RNA gene with alignment based on established secondary structures proved more useful for resolving the relatedness of this group [36, 56]. An independent assessment of a 684bp section of the mitochondrial cytochrome oxidase II region [57] found the COII useful in resolving most Australian and Oriental anophelines at the species level, but limited in resolving the known members in the *An. punctulatus* group. However, most phylogenetic studies of the group do consistently reveal two main clades, one containing all the *An. farauti*-like species (all-black proboscis) except *An. farauti* 4, which

appears in a second clade with *An. punctulatus* and *An.* species near *punctulatus* (all of which can display a half-black, half-white proboscis) [31, 58] (see Fig. 2 and Table 2). *Anopheles koliensis* is positioned either basal to all species in the COII tree or between the *An. farauti* and *An. punctulatus* clades in the rDNA trees, neither of which branches showed strong support.

The same evolutionary mechanisms that led to the existence of these species have also produced a number of genetically distinct populations within each species that may differ in behaviour and in their potential to transmit malaria parasites. For example, recent investigations have revealed that genotypes of *An. hinesorum* exist in the Solomon Islands that do not appear to bite humans while in other parts of this species' range, there are distinct genetic populations that are anthropophilic and are known to transmit malaria [51, 54, 59]. This study revealed restricted gene flow throughout *An. hinesorum*'s distribution and distinct differences in malaria vectoring potential and demonstrates the importance of detailing how species' populations connect to each other through population genetic studies – particularly in light of the design and efficacy of any control strategy [60].

2.2. *Anopheles (Cellia) longirostris* complex

The morphospecies *Anopheles longirostris* Brug is widespread throughout the coastal and inland lowland regions of New Guinea. Subsequent analysis of this morphospecies using both mtDNA and the rDNA ITS2 from 70 sites in PNG revealed up to nine distinct species that appear reproductively isolated at the rDNA locus [61]. Most of these putative species also exist as distinct mtDNA COI lineages and have been designated A, B, C1, C2, D, E, F, G, H [61]. Fig. 6 displays the phylogenetic study and molecular diagnostic developed with the same *Msp* I PCR-RFLP method as used for the *An. punctulatus* group. Of note, the species designated C1 and C2 produce the same ITS2 PCR-RFLP banding profile but curiously display different ITS2 copy variant organization. Where C1 is uncommon and extant only in the Western Province of PNG to date, species C2 appears to be the most common and widespread species in the group [61]. Thus the molecular diagnostic discrimination of C1 and C2 may only be problematic south of the central highlands in PNG's Western Province. However, species C1 may exist north of the central highlands. As it is only a recently recognized cryptic species group, little is now known about each species' biology and ecology and malaria transmission potential.

2.3. *Anopheles (Cellia) lungae* complex

Initially described by Belkin [20], the *An. lungae* group members show a distribution throughout the highly malarious Solomon Islands and Bougainville to the north. Belkin described three distinct morphological forms – *An. lungae*, *An. solomonis* and *An. nataliae* [20] – and variation among geographical populations was also noted. [20]. The three species have white scaling on the halteres which readily separates them from the members of the *An. punctulatus* group which occur in the Solomon Islands [20]. Within the *An. lungae* complex the members can be separated using proboscis morphology though there is some overlap between the species with this character and this method is not reliable. A molecular diagnostic has been developed for the three species based on a *Msp* I digest of the ITS2 (Fig. 7).

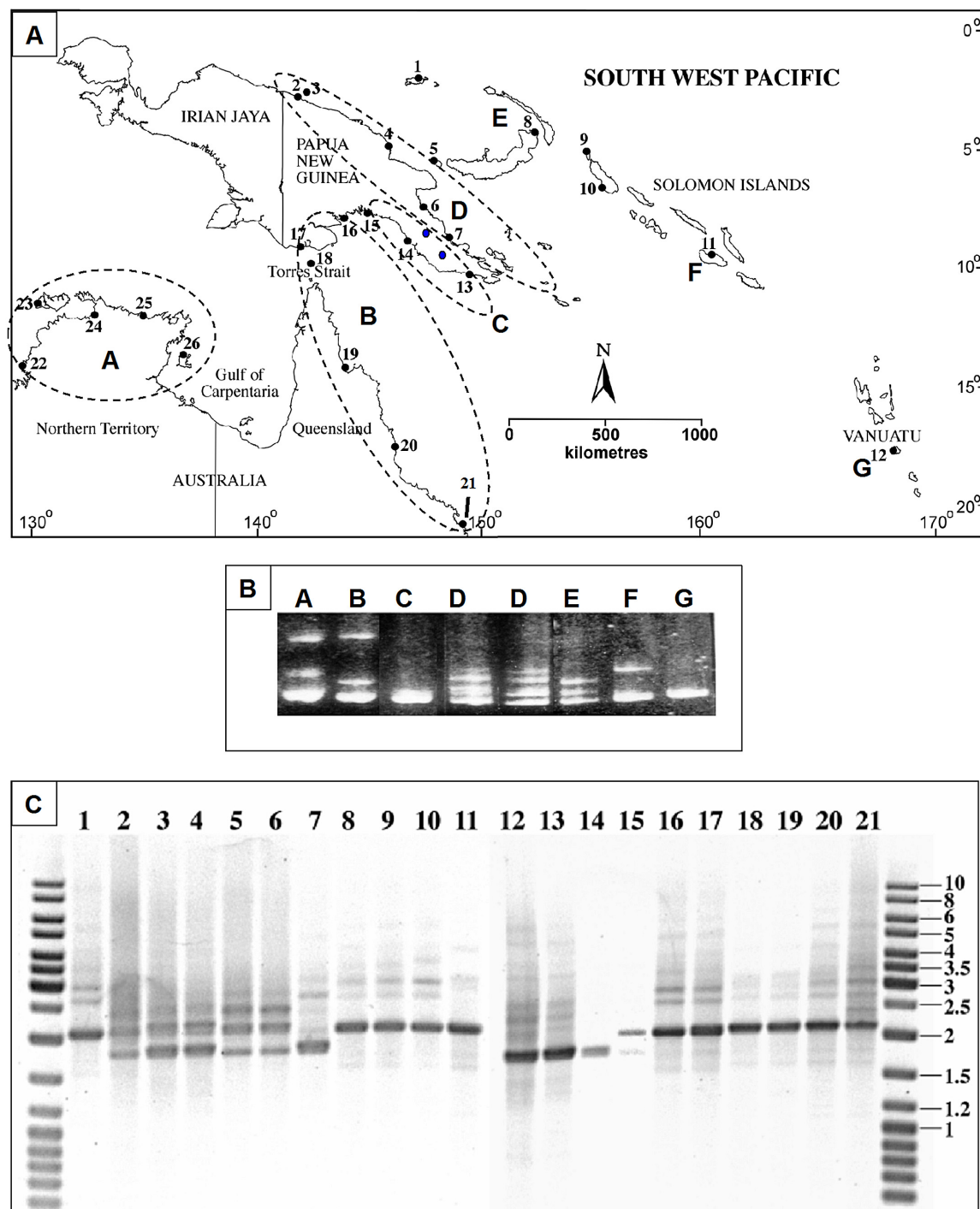


Figure 5. The rDNA genotypes of *An. farauti*. **Panel A** shows a map of southwest Pacific and the 21 *An. farauti* collection sites. Dotted circles represent the distribution grouping of ITS2 PCR heteroduplex profiles (genotypes) that appeared in native acrylamide gels shown in **Panel B** (samples 22-24 not shown). **Panel C** is an agarose gel showing individual *An. farauti* ITS1 PCR products with lanes representing collection sites on Panel A. Intragenomic size variation is evident between collection sites and in most cases individuals showed the same ITS1 and ITS2 heteroduplex profiles, exceptions were found in some sites on the north coast of PNG where rDNA profiles are highly polymorphic. This coastally restricted species shows remarkable rDNA turnover throughout its distribution. Cloning and sequencing ITS2 copy variants revealed no phylogenetic information, however the longer ITS1 (up to 2.5kb) revealed a robust phylogenetic signal resolving genotypes into regions [52].

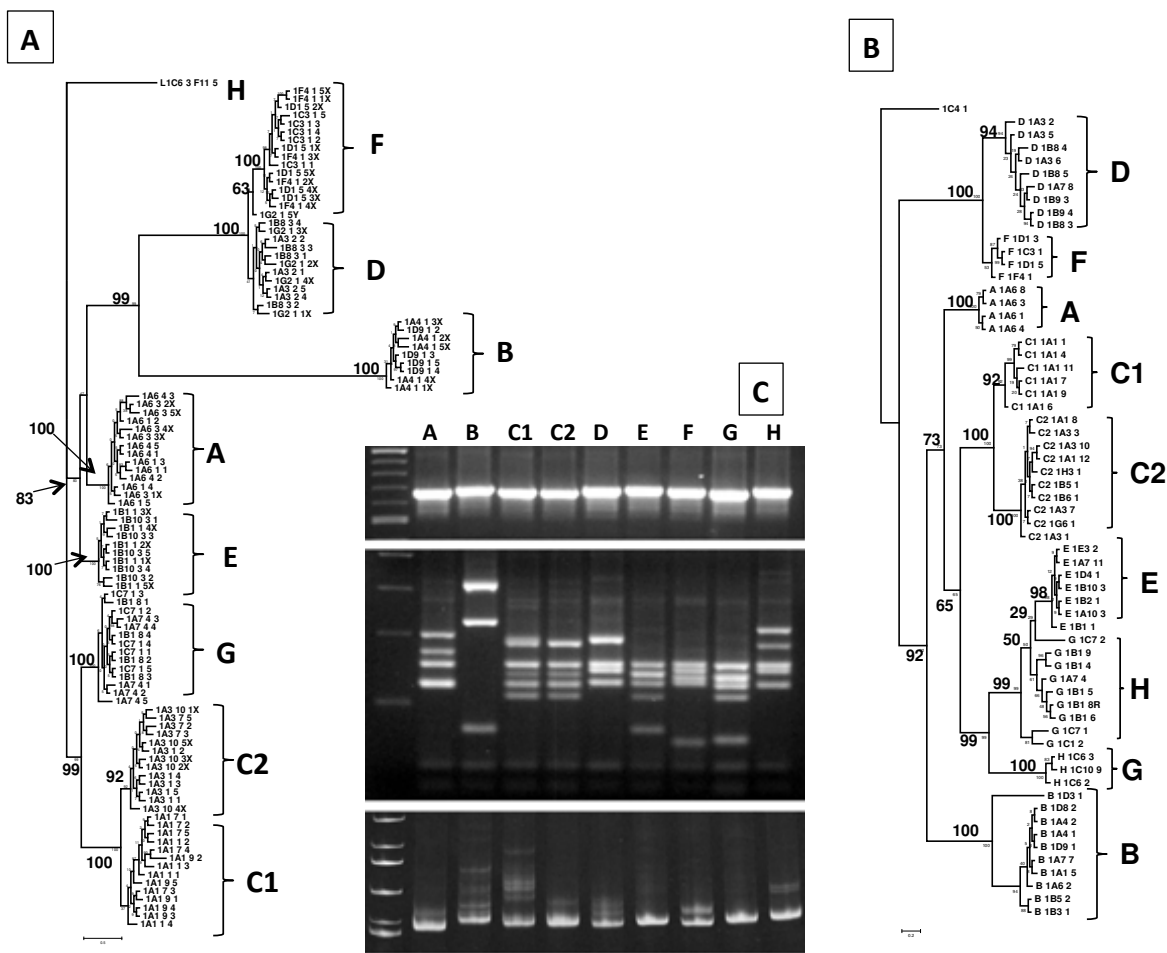


Figure 6. The discovery of nine cryptic species within mosquitoes identified morphologically as *Anopheles longirostris* from PNG. Phylogenetic assessment of *A. longirostris* based on cloned ITS2 DNA sequence from PCR products (**Panel A**) and directly sequenced mtDNA COI PCR products (**Panel B**) reveal nine distinct lineages. Bayesian posterior probabilities (converted to percentage) are shown as branch support values above 70%. **Panel C:** The molecular diagnostic developed revealed nine ITS2 genotypes of *A. longirostris*. Panel C-top, uncut ITS2 PCR products; Panel C-middle, ITS2 PCR products cut with *Msp* I and run through a 3% agarose gel; Panel C-bottom, ITS2 PCR products run through a 7.0% 7 acrylamide gel revealing individuals within interbreeding populations contained fixed copy variants, suggesting reproductive isolation at the rDNA locus. Only the RFLP profile for genotype C showed two distinct heteroduplex profiles (designated C1 and C2) thus revealing the presence of two independently evolving ITS2 genotypes.

2.4. *Anopheles* (*Anopheles*) *bancroftii* group

Two morphological species were initially described in the *Anopheles bancroftii* group based on wing fringe patterns –*Anopheles bancroftii* Giles, and *Anopheles pseudobarbirostris* Ludlow [63] – although some confusion as to the distributions of these two morphotypes existed. The ITS2 PCR-RFLP method using the enzyme *Msp* I identified four distinct ITS2 genotypes designated A, B, C and D [39]. ITS2 DNA sequence analysis of this group revealed intragenomic sequence copy variants existing in individual mosquitoes that assist in the identification of these four ITS2 genotypes (Fig. 8). For example, genotype C could be interpreted as a combination (hybrid) RFLP profile between genotypes A and B, however both DNA sequence analysis and intragenomic ITS2 copy variant studies revealed the presence of four independently evolving

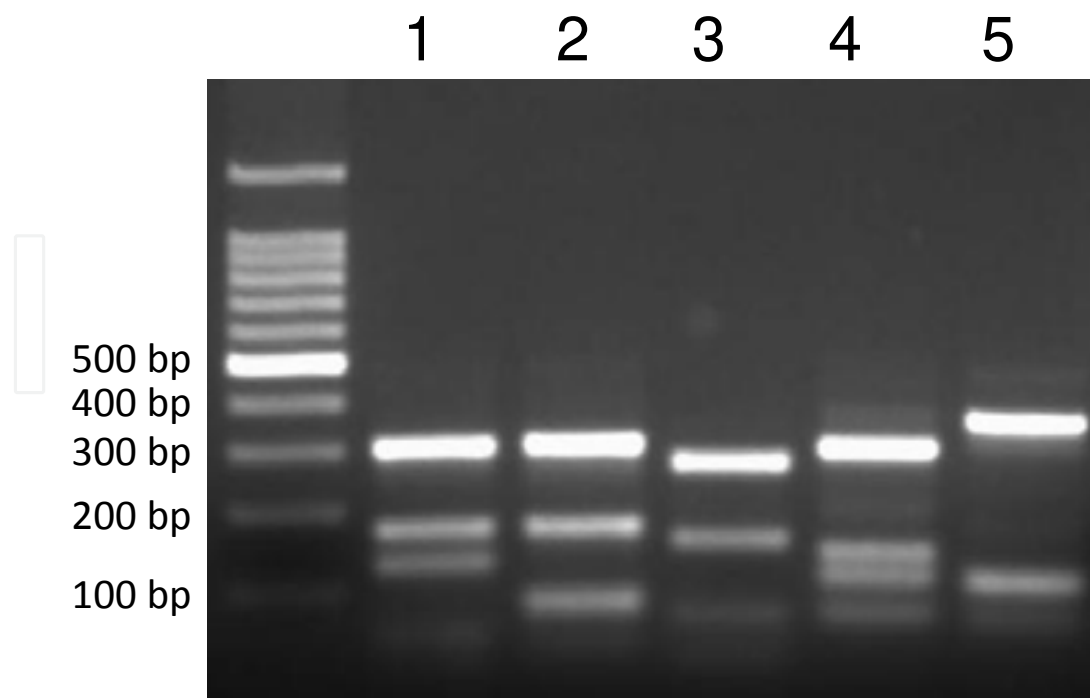


Figure 7. Molecular diagnostic for *Anopheles* species collected in Santa Isabel Province in the Solomon Islands based again on an ITS2 PCR-RFLP using *Msp* I [62]: Lanes 1-2 isomorphic species *An. farauti*, *An. hinesorum*. Lanes 3-5 are cryptic the members of the *Anopheles lungae* complex that exist in the Solomon Island: Lane 3, *An. nataliae*; Lane 4, *An. lungae*; and Lane 5, *An. solomonis*.

ITS2 genotypes with cloned ITS2 sequences showing little phylogenetic information [39]. No correlation was identified with the wing fringe characteristics initially used to identify *An. bancroftii* and *An. pseudobarbirostris* with any of the four genotypes. The distribution of these ITS2 genotypes (putative species) has been further investigated [64], indicating distinct distribution for genotypes A, B, and D. Genotype C is sympatric with B and D without evidence of hybridization, suggesting these genotypes are reproductively isolated and likely biological species. Confirmation of this hypothesis using other nuclear genetic markers is needed. Thus genotype C is sympatric with B and D without evidence of hybridization, suggesting these genotypes are reproductively isolated and likely biological species. Confirmation of this hypothesis using other nuclear genetic markers is needed.

3. Species distribution, biology and vectorial status

3.1. Primary vectors

Three species – *An. farauti*, *An. koliensis*, and *An. punctulatus* – are considered the primary vectors of malaria in the region. All are widely distributed and can occur in high densities (Fig. 9). They readily feed on humans, and all have been found infected with human malaria parasites.

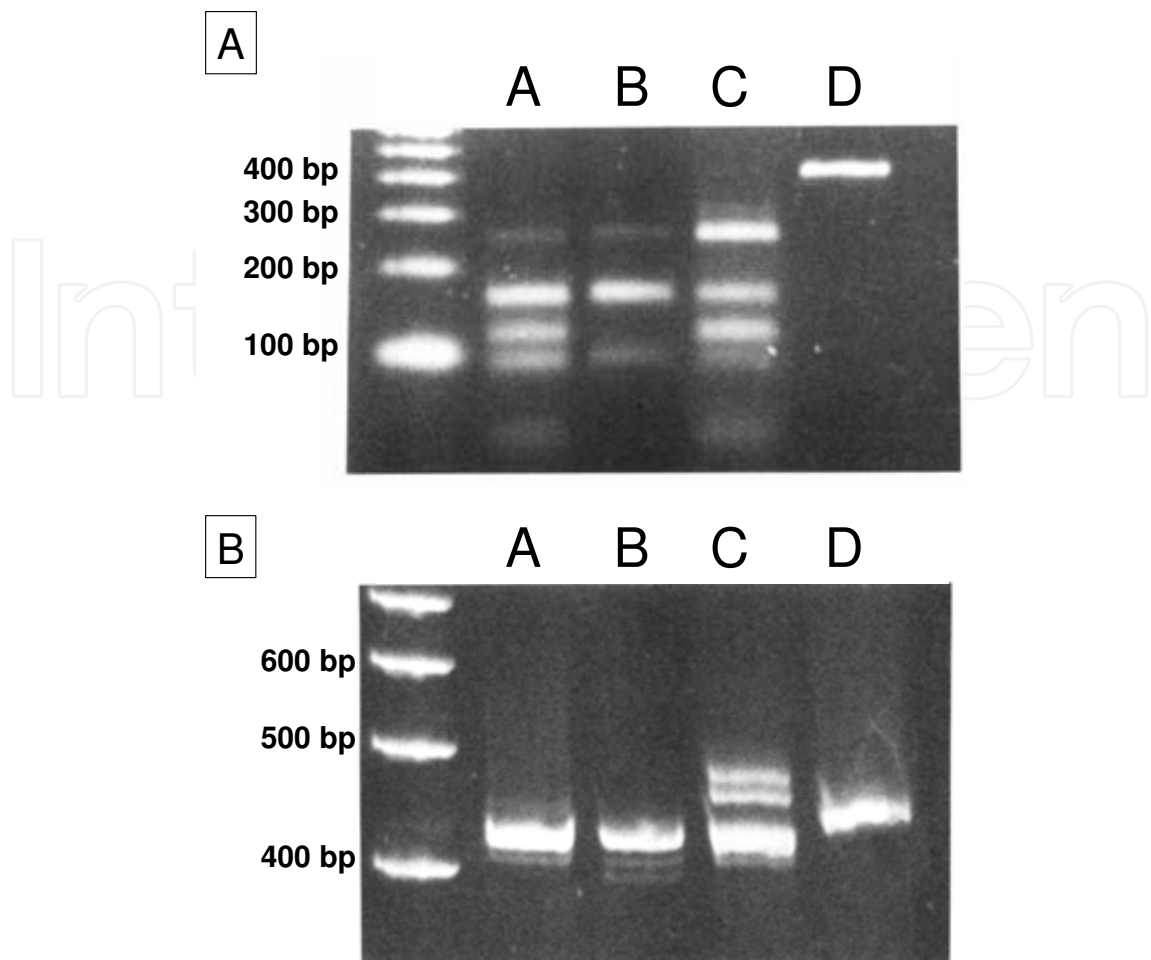


Figure 8. Molecular diagnostic for the cryptic species in the *An. bancroftii* group. **Panel A** are *Msp* I cut ITS2 PCR-RFLP profiles of *An. bancroftii* electrophoresis run through a 3.0% agarose gel. First lane on the left is a 100bp marker. Lanes 2-5 are the RFLP of genotypes A-D with genotype D revealing no *Msp* I restriction sites and the full length of the PCR product (all genotypes produce a 400bp PCR product). **Panel B** are the same PCR products electrophoresed through a 7.0% acrylamide gel that is sensitive to double stranded secondary structure. Lanes A, B and D show a single band for the amplified ITS2 (homogenized single sequence or homoduplex). Lane 4 is genotype C showing both a homoduplex (bottom band) and two heteroduplex products (misspairing in double-stranded duplex alters secondary structure retarding migration). Lane 5 is genotype D that migrates slower due to differences in the secondary structure duplex and not sequence length.

Anopheles farauti has the widest distribution of all the anopheline fauna of the region, occurring in the Moluccas, on New Guinea and its associated islands and archipelagos, in northern Australia, throughout the Solomon Islands and Vanuatu. *Anopheles farauti* has been incriminated as a vector of malaria throughout this range [59, 65-68]. It is a coastal species, whose larvae tolerate brackish water [28, 69], with preferred breeding sites ranging from small ground pools to large coastal swamps and lagoons formed where runoff to the sea is blocked by sand bars (Fig. 10 E). These large sites are ubiquitous along the coastline throughout the region [58, 62] and are often associated with human habitation. Due to their size, they can support high population numbers [62, 70]. *Anopheles farauti*'s ability to breed in brackish water has facilitated its dispersal across the myriad tiny islands throughout the region [20, 71].

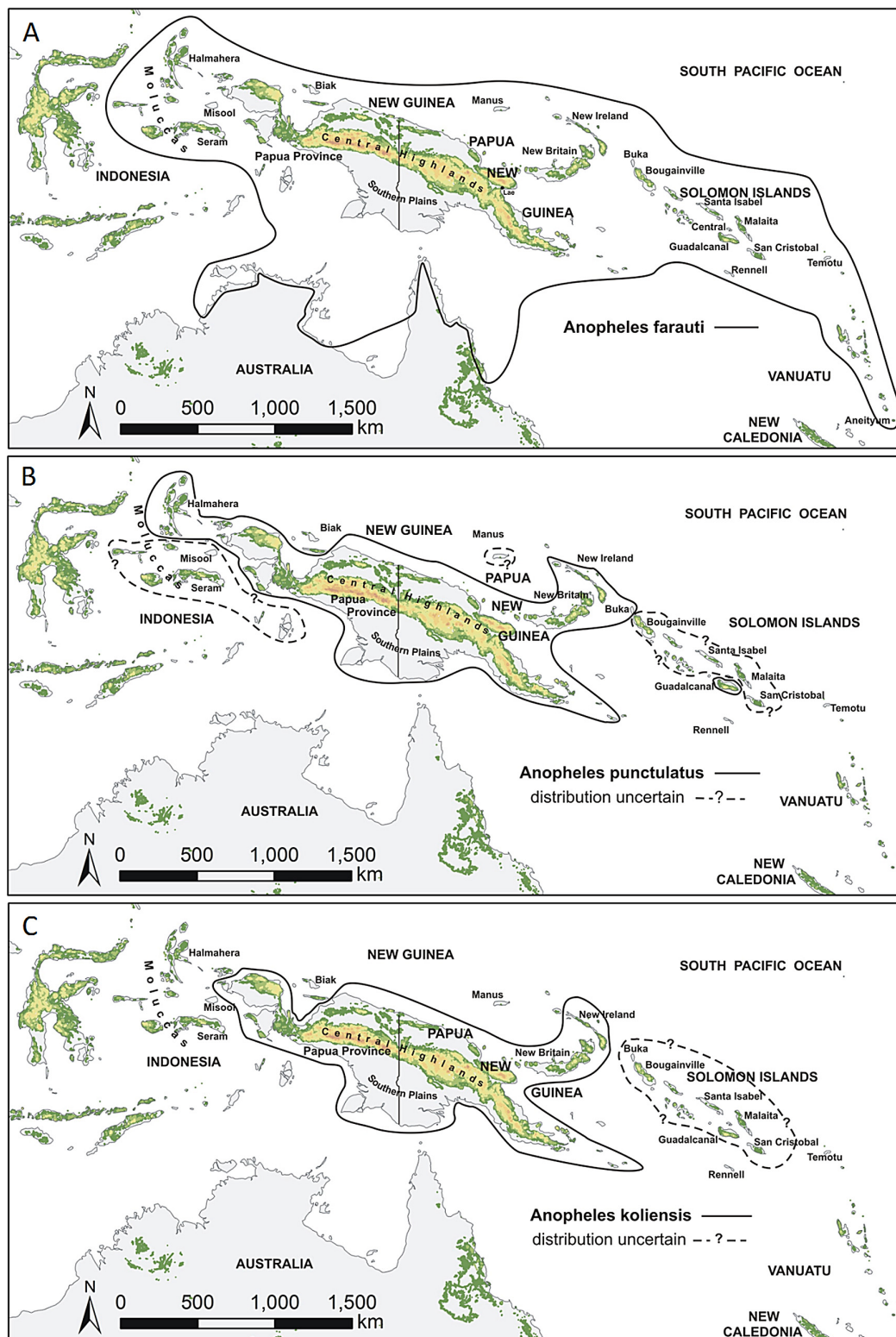


Figure 9. Known distributions of the three main species of the *An. punctulatus* group. **Panel A** is *An. farauti* which throughout its distribution is a coastally restricted species rarely found more than 5 km inland. **Panel B** is *An. punctulatus* which is a fresh water species that exists both coastal, inland and at elevation >1500m. **Panel C.** *An. koliensis* is a lowland inland and coastal species.

In PNG, the Solomon Islands, and Vanuatu, where extensive sampling has occurred and the mosquitoes' distribution is well understood, *An. farauti* is known to exist as several genotypes [53]. These genetically distinct populations are separated by overt barriers: climate disjunction between the northern continuous wet and southern monsoonal region in the Southern Plains of New Guinea (see Fig.1), the central highlands in New Guinea; and sea gaps between New Guinea and Manus Island, the Solomon Islands and Vanuatu [58]. All genotypes appear to have similar behaviours and are malaria vectors wherever they occur.

Given that *An. farauti* remains the dominant species collected in coastal villages, past reference to their biology and behaviour prior to identification using molecular techniques is probably still valid. *Anopheles farauti*, while readily feeding on humans, will also feed on other animals, and anthropophilic indices can be quite low in villages where domestic animals, primarily pigs and dogs, are abundant [27, 67]. Populations of this species were found well outside the flight range of human habitation, indicating that this species will readily feed on native birds and animals [31]. The longevity of this species appears quite variable; in the Solomon Islands province of Temotu the proportion of the population that was parous was 0.42 [70] while in Central Province it was 0.76 (T. Russell, unpublished data). In New Guinea, values ranged from 0.58 in Jayapura [65] to 0.49 in Madang, [27] and 0.73 in the D'Entrecasteaux Islands [66]. It will readily enter houses to feed but is primarily exophilic, leaving the house on the night of feeding to rest outdoors [65, 66].

Anopheles punctulatus has been recorded from the Moluccas, New Guinea, and the larger islands of Manus, New Britain, New Ireland and Buka – but it does not appear to be present on Bougainville Island [48, 72]. During faunal surveys conducted in the early 1970's, *An. punctulatus* was found on all the main islands in the Solomon Islands except Temotu Province [73]. It was found on Malaita in 1987 [74] and on the north coast of Guadalcanal in 1998 [51]. However, recent surveys of Santa Isabel and Central Provinces failed to find this species (62, T. Russell, unpublished data). In New Guinea, it is mainly found in inland lowland regions but is also common in the foothills of central ranges and in the intermountain highland valleys [8, 31, 75]. Its natural larval habitats are rock pools, pools in rivers and streambeds, and pools along the margins of these waterways. It is a highly invasive species and will readily invade sites created by human activity such as wheel ruts in roads, pools in walking tracks, hoof and foot prints, pig wallows and shallow drains around village houses (Table 3, Fig. 10 A) [31, 76]. These sites all have a clay or gravel substrate; are small or transient and are maintained only by regular rainfall; they lack established aquatic fauna and flora; and they have little or no debris.

Given that many rural communities throughout the region are connected by unsealed dirt roads, these thoroughfares – along with roads and construction associated with logging and mining activities – have created both extensive larval sites for this species and the corridors along which it can move. *Anopheles punctulatus* has adapted to these small transient sites with eggs that can survive desiccation for several days, a short larval stage (relative to other species) and highly synchronized larval development [76, 77]. A preference for transient sites binds *An. punctulatus* to areas where the soil contains clay and the rainfall is perennial. Where these conditions exist it can occur in high densities [65]. It is considered the most anthropophilic of

all the members of the *An. punctulatus* group [67, 78], and is a late night feeder with a feeding peak between midnight and 2am [79].

Of the three primary malaria vectors in the southwest Pacific, *An. punctulatus* is the most long lived [80]. It is a dangerous vector responsible for maintaining holoendemic transmission rates in a number of areas [78]. It has been incriminated as a malaria vector throughout its range [8, 59, 65, 67, 68, 75].

Anopheleskoliensis has a more complex distribution. It is found throughout New Guinea but not in the Moluccas; it occurs on New Britain and Buka Islands, but not on Bougainville; it was found on all the main islands in the Solomon Islands except those of Temotu Province [31, 72, 73, 81]. However, it can no longer be found in the islands of Santa Isabel, Guadalcanal, and Buka [48, 51, 62]. It was possibly eliminated from most islands in the Solomon Islands by IRS with DDT, with the last occurrence reported on the island of Malaita in 1983 [74]. Predominantly an inland species of the lowlands and river valley flood plains below 300m, its main larval habitats are wheel tracks, drains, natural ground pools, and swamps (Table 3, Fig. 10) [31]. Molecular investigations suggest there may be as many as three independently evolving rDNA genotypes (putative species) within this taxon in the Madang/Maprik areas alone [82], and possibly also elsewhere in PNG (N. Beebe, unpublished data). While *An. koliensis* will feed on pigs and dogs, it prefers humans where available and human blood indices of 0.85 and 0.95 have been recorded [27, 67]. It tends to feed late in the night with a peak biting time similar to *An. punctulatus* [65, 79]. In the village of Entrop, Papua Province, peak biting was at 7pm in DDT sprayed villages most likely due to the selection pressure to avoid the DDT, where in Arso (~50km away), which was not sprayed, peak biting was around midnight [65].

It is a moderately long-lived mosquito with parity rates ranging between 0.52 and 0.75 [65, 83]. It has been incriminated as a vector throughout its range [8, 59, 65, 67, 68]. Along with *An. punctulatus*, it is responsible for maintaining holoendemic transmission in a numbers of areas in New Guinea [65, 78].

3.2. Secondary vectors

A number of species have been found infected with human malaria sporozoites throughout the southwest Pacific, but because they have limited distributions or are relatively uncommon, they are considered secondary vectors.

Anopheleshinesorum (formally *An. farauti* 2) is almost as widespread as *An. farauti*, being found from the Moluccas throughout New Guinea, and on Buka and Bougainville Islands; it is also thought to occur in New Britain, New Ireland, and Manus [31, 48]. In the Solomon Islands, it was found on the islands of Santa Isabel, Central Province and the north coast of Guadalcanal, but does not occur in Vanuatu [51, 62, 70]. Any understanding of its distribution is limited by the paucity of faunal surveys in this region, and it is likely that it will be found on all the main islands in the Solomon Islands except Temotu. In Papua New Guinea this species is most frequently found in lowland inland river valleys and flood plains – however it also occurs on the coast and on small offshore islands [31].

Several genetically structured populations were found within *An. hinesorum* [54], with the genotypes found in Buka and Bougainville in PNG and in the Solomon Islands provinces of Santa Isabel, Central, and Guadalcanal being highly zoophilic and rarely biting humans [35, 48, 51, 62]. On mainland PNG, *An. hinesorum* readily bites humans; it was the most common anopheline found throughout the Southern Plains where it can occur in high densities [59]. It has also been found in the highlands of the central highlands (up to 1740m), though it is less common in this region. This is also the case north of the central highlands, possibly due to competition from other species such as *An. farauti* 4 and *An. koliensis*, which also occur in this region and share similar larval habitats. *Anopheles hinesorum* has been incriminated as a vector in this northern New Guinea region [59].

Anopheles hinesorum oviposits in a range of water bodies, both natural – ground pools, swamps and the edges of streams; and rivers – and human-made drains and ditches, wheel ruts and pig wallows (Table 3, Fig. 10) [31]. On Santa Isabel larvae were found in small, shallow, wheel ruts. These transient sites – turbid, with a clay substrate, and devoid of any vegetation – are, at least in Papua New Guinea, normally the exclusive habitat of *An. punctulatus*, but *An. hinesorum* now appears to occupy this niche in the Solomon Islands [62].

Species	Larval Habitat ¹ - incidence (%)								Totals
	Transie nt pools (A)	Ground pools (B)	Pig wallows (C)	Wheel tracks (D)	Swamp brackish (E)	Swamp fresh (F)	Edge of streams (G)	Drains earthen (H)	
<i>An. punctulatus</i>	115 (49.7)	41 (17.7)	15 (6.5)	34 (14.7)	0	2 (0.8)	3 (1.3)	21 (9.0)	231
<i>An. farauti</i>	7 (4.5)	48 (30.7)	1 (0.6)	22 (14.10)	43 (27.5)	2 (1.2)	12 (7.7)	21 (13.40)	156
<i>An. koliensis</i>	5 (7.9)	17 (27.0)	4 (6.3)	15 (23.8)	0	2 (3.1)	2 (3.1)	18 (28.5)	63
<i>An. hinesorum</i>	70 (18.7)	141 (37.7)	7 (1.8)	41 (10.9)	12 (3.2)	18 (4.8)	23 (6.1)	52 (13.9)	374
<i>An. farauti</i> 4	0	2 (25.0)	2 (25.0)	1 (12.5)	0	0	0	3 (37.5)	8
<i>An. farauti</i> 6	0	2 (33.3)	0	0	0	1 (16.7)	0	3 (50.0)	6
<i>An. bancroftii</i>	0	0	0	0	0	7 (70.0)	2 (20.0)	1 (10.0)	10

letters after habitat type correspond to illustrations in Fig. 10

Table 3. Larval habitats of some primary and secondary vectors of malaria in the Australian Region.

Little is known about this vector's behaviour with regards to malaria transmission although in northern PNG it appears that human feeding activity peaks early in the evening and then declines through the rest of the night [82].

Anopheles farauti 4 has been found throughout the inland lowland river valleys and flood plains north of the central highlands in PNG [31, 82]. In some locations it is very abundant, and in villages inland from Lae it can comprise up to 90% of the night-biting catch [31]. It readily utilizes larval sites created by human activity – pig wallows, drains, and wheel ruts (Table 3, Fig. 10) where it was commonly found in association with *An. punctulatus* and *An. koliensis*. It is a vector throughout its range [59, 82]. Little is known of its behaviour mainly due to the fact that there are no reliable morphological characters that separate it from *An. hinesorum* and *An. koliensis*, the two species with which it is commonly sympatric.

Anopheles farauti 6 is restricted in its distribution to the intermontane plains and upland valleys of the highland regions (>1000m, ranging to the highest points of 2000m) of New Guinea. It has adapted to the cool moist climate that prevails at these altitudes and in this habitat it is quite common. It is noticeably larger than any other members of the *An. farauti* complex [31, 84]. In 1960, Peters and Christian [7] found this large *An. farauti* to be the most common anopheline biting humans in the Waghi Valley in the highlands of PNG and recorded sporozoites in 2.2%. It was the most abundant anopheline in human biting catches in the Baliem Valley (Wamena, at 1,500m) in the central highlands of Papua Province [31]. *An. farauti* 6 likely plays an important role in malaria transmission within this restricted range.

Anopheles farauti 8, the most recent member of the *An. farauti* complex to be recognized, has to date only been found in the inland lowland areas on the east side of the Gulf of Papua in PNG [37]. However, given that this species has an ITS2 RFLP identical to *An. farauti*, it may have been confused with this species in past faunal surveys and its distribution may be more extensive than is currently known. Very little is currently known about this species other than that specimens infected with human malaria parasites have been found [31].

Anopheles longirostris s.l., now known to be a complex of nine species, [61] is found only on the island of New Guinea. It has a wide distribution below 1000m [31, 81], but has been recorded in large numbers only in a few areas. Its generally low abundance may be due to its preference for jungle pools associated with dense vegetation for oviposition. Behavioural studies have found it to be zoophilic in some areas [27] and anthropophilic in others [64] and these differences in behaviour may possibly be explained by the presence of cryptic species, each exhibiting different host-feeding preferences [61]. Little is known about the biology of these species and the individual role that each species might play in malaria transmission. It has been incriminated as a vector of malaria in the Southern Plains and north of the central highlands in PNG [59, 75].

Anopheles bancroftii s.l. has a wide distribution throughout New Guinea [64, 81]. It is now known to be a species complex containing four independently evolving genotypes [39], although its status with respect to *An. barbiventris* is unknown. *Anopheles bancroftii* A is found throughout northern Australia and the Southern Plains of PNG where it is common, occurring in large numbers and readily biting humans. Genotype B occurs in Papua south of the central highlands

and genotype D occurs in the inland river valleys north of the central highlands. The range of *An. bancroftii* C overlaps with genotypes B and D. genotypes B, C, and D are rarely collected near the coast and appear to prefer inland, lowland, river flood plains below 150m. In PNG the members of the *An. bancroftii* complex are rarely found anywhere in large numbers, except for the Southern Plains. Members of the complex have been incriminated as vectors of malaria at only a few locations [59, 75]. Larval habitats are mainly large permanent water bodies such as fresh water swamps and lagoons (Table 3, Fig.10 F). Nothing is yet known about the biology or behaviour of any of these putative species.

3.3. Possible vectors

There are several *Anopheles* species found throughout the southwest Pacific that feed on humans but are not very abundant and have limited distributions – in most cases, little is yet known about their biology or behaviour. These include *An. meraukensis*, *An. novaguinensis*, *An. torresiensis*, and *An. hilli* – all of which are found only on the Southern Plains of New Guinea (Fig. 1). All four species are common in northern Australia where a similar climate type also prevails [31, 85, 86]. In Australia these four species will readily bite humans, but in PNG nothing is known about the biology of these species except that *An. hilli* can occur in large numbers, will feed on humans, and will enter houses to do so [87]. *Anopheles hilli* was incriminated as a vector of malaria in Australia during a *Plasmodium vivax* epidemic in Cairns in 1942 [88]. These four species may be involved in malaria transmission but only as minor local vectors at best.

The members of the *Anopheles lungae* complex – *An. lungae*, *An. solomonis*, and *An. nataliae* – are endemic to the Solomon Islands where they are found on all major islands except Temotu, with *An. lungae* also being recorded from Bougainville [70, 89]. All three species have been recorded to bite humans and there is some circumstantial evidence incriminating *An. lungae* as a malaria vector [18]. On Santa Isabel, *An. solomonis* was found to be the dominant human biting anopheline in inland villages although they were also recorded biting pigs. This species fed outdoors, early in the evening (6pm-9pm) but was short-lived. In a sample of 221 mosquitoes collected via human landing catches, the proportion of parous was 0.33 [62]. No member of the *An. lungae* complex has been found infected with human malaria parasites although their human biting behaviour would make them possible vectors.

3.4. Non-vectors

Several *Anopheles* species present in the southwest Pacific are known not to feed on humans and this zoophilic behaviour precludes them from being vectors of malaria. These species include *An. annulipes* L and *An. annulipes* M, which are part of the *An. annulipes* complex – the members of which are widespread throughout Australia [90]. *Anopheles annulipes* L is found in a small enclave of monsoonal climate, which exists along the southern coast of Papua around Port Moresby, and within this limited distribution, it is quite common (Fig. 1). *Anopheles annulipes* M is a highland species common in intermontane valleys above

1000m [64]. Both species are readily found as larvae but are rarely collected feeding on humans [7, 64].

Anopheles sirenicus (formally *An. farauti* 7) is endemic to the Solomon Islands, being recorded only on Guadalcanal. Larvae are commonly collected but the adults have never been recorded as biting humans [35, 51].

Anopheles sp. near *punctulatus* is an uncommon species with a patchy distribution restricted to the upland valleys of the central highlands in Papua New Guinea [31]. Nothing is yet known of its biology though it appears to have little association with humans.

Several species that occur in the region have limited distributions and are too uncommon to play any significant role in malaria transmission. These species include *An. papuensis* and *An. farauti* 5, two rarely recorded species from the highlands of PNG; *Anopheles clowi*, found on only two occasions since 1946 [19, 91]; and *An. rennellensis*, found only on the malaria-free island of Rennell in the Solomon Islands [25].

3.5. Oriental species

Five anopheline species – *An. annulatus*, *An. kochi*, *An. indefinitus*, *An. vanus*, and *An. vagus* – are Oriental species found as far east as the Moluccas Islands, which borders the Australian Region [18]. Two others – *An. karwari* and *An. subpictus* – have made substantial dispersals into New Guinea. While *An. tessellatus* has also been recorded in Papua Province and more recently in the Jayapura area (N. Lobo, unpublished data), it is not considered a vector in the Australian Region.

Anopheles karwari was first reported in Jayapura in the 1930s where it was believed to be relatively common [92]. In PNG, the first record was from Maprik in 1960 [78], with subsequent confirmation by Hii and colleagues in 1997 [93] who also recorded it from the Maprik area where it made up 14% of the anophelines collected. Its distribution appears to be restricted to inland lowlands, and to foothills (up to 1000m) on the north side of the central highlands in PNG [31]. Nothing is known of its larval habits in PNG, but in Papua Province it was recorded from the edges of slow-moving watercourses, seepages, grassy pools, wheel ruts and hoof prints. *An. karwari* was first incriminated as a vector in 1955 in Papua Province [94]; in PNG it was positive for sporozoites in the Watut Valley inland from Lae [64], and in Maprik [75]. *Anopheles karwari* can be abundant but given its limited distribution, it is considered a secondary vector.

Anopheles subpictus occurs in the Moluccas, in Papua Province, and has been found on the islands of Biak and Misool. It has been found in several isolated populations in Papua New Guinea but only appears to be well established and common along the south coast of PNG from the Gulf of Papua to the D'Entrecasteaux Islands [64, 95]. It is a brackish water breeder and so is restricted to the coast. There are records of it biting humans and being infected with malaria at Bereina west of Port Moresby [95-97]. Apart from the population along the southern coastline of PNG, *An. subpictus* is uncommon with a limited distribution, and so is considered only a secondary vector.



Figure 10. *Anopheles* larval sites as described in Table 3. **Panel A**, transient pool; **Panel B**, ground Pool; **Panel C**, pig wallow; **Panel D**, tyre track; **Panel E**, brackish swamp; **Panel F**, fresh water swamp; **Panel G**, edge of stream; **Panel H**, Drain.

4. Vector control

The strategy behind the use of indoor residual spraying (IRS) and insecticidal treated bed nets (ITNs) is to deliver insecticide to vectors which have entered the house to obtain a blood meal. Given that a female mosquito feeds every second or third night, it will seek a blood meal at least 3 to 5 times during the duration of the extrinsic incubation period, allowing 3 to 5 opportunities to contact the insecticide associated with IRS and ITNs before it develops sporozoites in the salivary glands. Ideally, for IRS and ITNs to successfully control malaria,

the vector should exhibit the following behaviours: a) be highly anthropophilic, b) feed indoors late at night when the humans are indoors, and c) rest on the insecticide treated surfaces of ITNs or IRS either before or after feeding.

The primary vectors in the southwest Pacific initially were reported to exhibit this type of behaviour to varying degrees. *Anopheles punctulatus* is the most anthropophilic of the three vectors [78, 98] and has a peak night-biting time around midnight [79]. *An. koliensis* is the next most anthropophilic and also feeds late at night [78, 79, 98]. On the other hand, *Anopheles farauti* is the least anthropophilic or most opportunistic species, and while it also had a peak feeding time around midnight, it starts feeding earlier in the evening at dusk [99] – when hosts are less likely to be inside or under nets. A pattern of early evening blood feeding was reported in the 1960's [65], 1970's [100] and 1980's [101]. While all species will readily enter houses to obtain a blood meal, none remain inside houses after sunrise [65, 99]. Thus while ITNs and IRS control may well be efficacious against late night biting *An. punctulatus* and *An. koliensis*, adaptation of *An. farauti* to feed primarily early in the evening [100] may minimize the opportunities to contract insecticides and thereby circumvent control efforts with IRS and ITNs.

With the implementation of the eradication program and subsequent control programs using DDT with IRS, populations of *An. punctulatus* were reduced to the point where adults and larvae of this species were virtually impossible to find. This was not an isolated occurrence but was found across all areas where these programs were implemented and the behaviours of the vectors were studied: Arso and Entrop in Papua Province; Maprik and Wewak in PNG; Rabaul in the islands of PNG; and in the Solomon Islands [100-103]. *Anopheles koliensis* populations were also suppressed by IRS though the extent of this suppression varied: in Arso, the reduction was short lived, while in PNG it appeared to be more sustained and in the Solomon Islands this species may have been eliminated [65, 72, 100].

Where *An. farauti*, populations were suppressed by IRS, they returned to pre-spray levels after only a few years [100, 101]. In Wewak, on the coast from Maprik, this happened after the first spray round, and in the Carteret Islands IRS had little effect on the population density of *An. farauti* [72].

Slooff [65] studied the house-visiting behaviour of *An. farauti* and observed that fewer mosquitoes entered DDT sprayed houses compared to the unsprayed houses and that their feeding success was less in sprayed houses. Thevasagayam [104] found that >45% of indoor-feeding *An. farauti* in the Solomon Islands left the house before picking up a lethal dose of insecticide. Slooff [65] suggested that this behaviour was due to an irritant effect of the DDT, a phenomenon that has been understood for some time [105] and which appeared to be pronounced in *An. farauti*.

Studies into the failure of IRS to adequately control populations of *An. farauti* revealed a major shift in the biting time of this species (and to some extent in *An. koliensis* as well) following IRS [65]. Before IRS *An. farauti* commenced feeding at dusk and built up to a peak at midnight [66, 79]. However following IRS, the majority of feeding occurred between 6pm and 8pm [66, 100]. A typical example was New Britain in 1963 where *An. farauti* before IRS with DDT fed throughout the night with a peak at midnight, but after five spray cycles (across two years)

there was a distinct peak of feeding between 6pm-7pm, with 76% of feeding occurring before 9pm. [101]. It is common for the human populations in this region to spend the first hours of the night outdoors and so by feeding early in the night, *An. farauti* can obtain a blood meal without entering houses and being exposed to the insecticides used in IRS or ITNs. In the Solomon Islands this change in behaviour was believed responsible, in part, for the inability to interrupt transmission and the eventual failure of the eradication program [106].

This shift in biting time to early in the night appears fixed in some populations: when spraying was withdrawn, the early night-feeding pattern was maintained. In Temotu and Santa Isabel in the Solomon Islands, where DDT IRS was intensively applied during the eradication program of the early 1970s but only intermittently during the subsequent 35 years, *An. farauti* still displays the early night-biting pattern [62, 70]. In Temotu, with the resumption of a malaria elimination program in 2009 (based on the use of pyrethroids in IRS and distribution of ITNs), the early night-biting activity was further enforced with an increase in outdoor biting from 43% to 60% without any significant reduction in biting density post-intervention [70].

On Buka Island, in 1961 prior to spraying with DDT, *An. farauti*, *An. punctulatus*, and *An. koliensis* were all present. A post-spray survey conducted one year later found only *An. farauti* (see Spencer, unpublished report to the Department of Health, Malaria Control Program, Papua and New Guinea, 1961). DDT IRS on Buka continued for the next 20 years (40 spray rounds). Entomological surveys in 2000 failed to find *An. koliensis*; however at this time both *An. farauti* and *An. punctulatus* were abundant, indicating the reintroduction or recovery of the latter species. The night-biting pattern of *An. farauti* at this time showed the classical pre-spray pattern, that is, a rapid build-up in numbers from 6pm to a peak at midnight [48].

There were only a limited number of vector control strategies evaluated in the southwest Pacific in the decades following the cessation of the IRS-based elimination campaigns. While the DDT campaigns did not succeed in eliminating malaria, the campaigns were credited with the elimination of filariasis from the Solomon Islands where that disease was transmitted by the members of the *An. punctulatus* group [107]. Most of the subsequent vector control evaluations were trials of bed nets, either untreated or treated with pyrethroids. Trials evaluated entomological as well as parasitological impacts for malaria and/or filariasis as anophelines vector both of these parasitic diseases. A single-village longitudinal study of untreated bed nets in Madang Province of PNG showed that nets significantly reduced the human blood index of *An. punctulatus*, as well as the infection rates for the *Plasmodium falciparum* CS antigen and *Wuchereria bancrofti* for both early and late stage larvae [108]. On Bagabag Island of Madang, PNG, where *An. farauti* is the vector, one study [109] reported that users of untreated nets had significantly lower microfilariae and filarial antigen positivity rates than individuals not sleeping under bed nets, suggesting that nets were effective in limiting filariasis transmission by *An. farauti*.

The first study of permethrin treated nets in PNG reported significant reductions in the sporozoite rates in the *An. punctulatus* group in two villages as well as a significant reduction in *P. falciparum* incidence in children under the age of four years [10]. At the same time, Charlwood and Dagoro [110], working in a different part of PNG, found that permethrin-treated nets deterred members of the *An. punctulatus* group from entering houses. Prolonged

ITN use in PNG was associated with reduced sporozoite rates, a result hypothesized to be due to a reduction in mosquito survival [111]. Bockarie and Dagoro [112] reported that ITNs were more effective in protecting against *P. falciparum* in PNG and postulated that this was due to vivax-infected members of the *An. punctulatus* group feeding earlier than falciparum-infected mosquitoes.

In the Solomon Islands, ITNs had significantly greater impacts than IRS on vector infectivity and inoculation rates of *An. farauti* and *An. punctulatus*, however the reductions in the entomological inoculation rates were insufficient to effectively control malaria without additional interventions [68]. Later, Hii and colleagues [113] reported that ITNs in villages extended the length of the oviposition cycle by one day compared to DDT or untreated villages, and in 1993, Kere and colleagues reported a 71% reduction in biting rates of *An. farauti* on Guadalcanal, Solomon Islands following the introduction of ITNs but questioned the effectiveness of the nets given that people spend considerable time outside [114]. An analysis of facility-based data showed that both IRS with DDT and permethrin-treated ITNs are associated with reductions in malaria incidence and fever, while larviciding with temephos was not [115]. Recently, Bugoro and colleagues [70] found “little, if any, reduction in biting densities and no reduction in the longevity of the vector population” in Temotu Province of the Solomon Islands following the introduction of LLINs and IRS.

In Vanuatu, malaria was successfully eliminated from the island of Aneityum using a strategy of mass drug administration with pyrimethamine/sulfadoxine (Fansidar), and primaquine, ITNs and larvivorous fish. Falciparum malaria disappeared soon after the start of mass drug administrations [13]. The successful elimination was a function, most likely, of a small island population and the seasonality of transmission together with a high participation of the community in the mass drug administration. The impact of larvivorous fish was believed to be “probably marginal” due to the failure to find all breeding sites and the “incompleteness of predation”.

Interpretation of the impact of these interventions must consider the period when the studies were conducted as reports of changes in behaviours of the vectors (discussed earlier) are known to have occurred; the effectiveness of an intervention is not static but is also dependent on the vectors' behaviours (e.g., shifts toward early feeding and outdoor biting may reduce the effectiveness of ITNs and IRS, as was demonstrated by Slooff [65], Taylor [100] and Sweeney [101]). Resistance to pyrethroids (and the existence of knock-down resistance genes) has not yet been found in the few studies thus far conducted in the southwest Pacific [116]; however, 30% of *An. koliensis* in Papua Province, Indonesia, were found to be resistant to DDT [117].

There is now a renewed interest in malaria control with IRS and ITNs in the Solomon Islands and Vanuatu with elimination programs in some areas and intensified control in all other areas. At the most fundamental level, the intervention measures of IRS and ITNs both rely on the vector feeding late at night when people are indoors. As such, these tools have the potential to provide effective control of late night biting *An. punctulatus* and *An. koliensis*. However it is important to emphasize that this behaviour pattern is no longer universally demonstrated by *An. farauti*, the primary coastal vector in the southwest Pacific.

The early biting pattern of the widely distributed *An. farauti* will prevent mosquito control and malaria elimination where this species bites early and outdoors and thereby avoids insecticides in IRS and ITNs. Therefore, additional control measures that target the vectors outside houses are now urgently needed for these programs to achieve effective reductions in malaria transmission. Effective larval control may be feasible with species such as *An. farauti*. Unlike *An. koliensis* and *An. punctulatus*, whose larvae are found in small ground pools that will be difficult to locate and treat where the annual rainfall is >2000mm, the most productive larval sites of *An. farauti* are large permanent coastal swamps and lagoons (Fig. 10 E) [62, 70, 118]. Such sites are easy to locate, few in number and permanent, and thus more easily treated.

5. Conclusion

In 2007, the Bill and Melinda Gates Foundation challenged the malaria community to once again attempt to achieve malaria eradication. The failure of the previous campaigns was due, in part, to attempting to control many vector species with a single intervention that targeted vectors inside houses. Enhancing our chances of eliminating malaria in the southwest Pacific will require the implementation of novel interventions that target vectors based on our knowledge of their behaviours. However, basic knowledge about the biology and behaviours of some vectors and potential vector species in this region is limited. This knowledge gap must be filled before control strategies can be optimized to exploit the vectors' biological vulnerabilities to control measures. The basic parameters essential to understanding transmission such as feeding habits, host preference, longevity, frequency of feeding and seasonal abundance – which are essential for the selection of effective control strategies –, await discovery for many species. Additionally, we remain uncertain of the complete distribution of species, or the importance of the various genotypes that have been recognized to date in a number of taxon.

Significant advances in DNA technologies have enhanced our ability to both discover and identify cryptic species in the southwest Pacific. These technologies, coupled with immunological and molecular assays to detect malaria parasites in mosquitoes, have led to the resurgence in investigations to incriminate vectors and to characterize their behaviors. We now know that there are 13 species in the *An. punctulatus* group (not three); that *An. longirostris* is not one zoophilic mosquito but a complex that includes human-biting malaria vectors; and that *An. bancroftii* is a complex of at least four species (not one as previously thought), two of which are malaria vectors. New studies on species-specific bionomic trails are enabling us to understand the biological basis for how they might be affected by interventions. Because of recent technological advances and their application to field studies, our knowledge on the major vectors in southwest Pacific is much better understood and as a consequence we are now better positioned than ever to study the species in this region and to design and evaluate novel and effective interventions.

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